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Comparative Alpha-amylase Inhibitory Properties of the Leaf Extracts of *Petiveria alliacea* L.

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Authors' contributions

This work was carried out in collaboration between all authors. Author SMA designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors MAO, SA and FD managed the analyses of the study. Authors SMA and MAO managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Aims: The study was designed to determine the phytochemical contents and the inhibitory effects of the leaf extracts of *Petiveria alliacea* L. on alpha-amylase enzyme.
Study Design: Phytochemical Screening and *in-vitro* evaluation of alpha-amylase inhibitory effects of the leaf extracts of *Petiveria alliacea* L using starch-iodide and 3,5- dinitrosalicyclic acid assays.
Place and Duration: This work was carried out in the Chemistry Laboratory of the Department of Science Laboratory Technology (Chemistry Unit), School of Pure and Applied Science, Lagos State Polytechnic, Ikorodu and the Biochemistry Laboratory of the Nigerian Institute of Medical Research, Yaba, Lagos, Nigeria, between April and August 2016.
Methodology: *Petiveria alliacea* leaves was successively extracted with n-hexane, ethyl acetate, and ethanol by maceration for 72 h to obtain the respective extracts. Phytochemical screening of

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the extracts for the presence of secondary metabolites: tannins, alkaloid, flavonoids, cardiac glycosides, reducing sugar, saponins, and anthraquinones was carried out using standard methods. The percentage of alkaloids and flavonoids was determined using Harborne and Boham and Kocipaiabyazan methods respectively. *In-vitro* alpha-amylase inhibitory properties of the leaf extracts were carried out by starch-iodine and 3,5-dinitrosalicyclic acid reagent (DNSA reagent) assays using acarbose as the standard.

Results: The results showed alkaloids, cardiac glycosides were detected in all the extracts. saponins, flavonoids, anthraquinones and reducing sugars were detected in the ethyl acetate and ethanol extracts; steroids are detected in the hexane extract only; tannins and terpenoids were not detected in all the extracts. The percentage of alkaloids and flavonoids are 2.14±0.16% and 6.14±1.07% respectively. All the extracts showed positive Alpha-amylase inhibitory properties with percentage inhibition increasing with increase in concentration. starch-iodine and 3,5-dinitrosalicyclic acid reagent assays gave similar values for percentage inhibition of alpha-amylase at 50 and 100 µg/mL concentration of extracts in the hexane, ethyl acetate extracts and acarbose with slightly different values for the ethanol extract.

Conclusion: The results suggested *Petiveria alliacea* leaf extracts could reduce post-prandial glucose level in type 2 diabetes mellitus and both methods of assay are effective.

Keywords: Phytochemical screening; alpha-amylase inhibitory; starch-iodide assay; 3,5- dinitrosalicyclic acid assay; Petiveria alliacea.

1. INTRODUCTION

Medicinal Plants continually provides new therapeutic pathways in the treatment of diverse diseases and in the process they have acted as pointers to new drug development [1]. These medicinal properties are as a result of their diverse bioactive components (phytochemicals) known for their therapeutic properties [2].

The research into the management of diabetes mellitus is an on-going process considering the increasing number of people suffering from the disease yearly [3,4]. A major problem in type 2 diabetes mellitus is high post-prandial glucose concentration which is responsible for the various complications associated with the disease [5]. One effective way of tackling the problem is to inhibit the activities of digestive enzymes like alpha-amylase and others that are involved in the hydrolysis of starch hence reducing the concentration of sugar after meals in diabetic patients [5,6].

Petiveria alliacea L. (Phytolaccaceae) commonly known as "Guinea Hen weed" is a common tropical rainforest plant found in the South America and Africa [7]. It is sometimes called "garlic weed" because of the garlic odor-like smell of the root [8]. In folk medicine, the root decoction, the powder and leave infusion of *Petiveria alliacea* are used to treat several ailments especially as anti-inflammatory, antispasmodic, and anti-helminthic. It is also used as sedatives, to treat leukemia and for the treatment of hyperglycemia [8,9]. The hypoglycaemic activity of *Petiveria alliacea* (Guinea Hen weed) extracts in normoglycaemic and diabetic rat models was investigated [10,11] however, the alpha-amylase inhibitory property is yet to be investigated.

In this work, the phytochemical contents and the alpha-amylase inhibitory properties of the hexane, ethyl acetate and ethanol leaf extracts of *Petiveria alliacea* L were investigated.

2. MATERIALS AND METHODS

2.1 Collection and Authentication of Plant Materials

Fresh leaves of *Petiveria alliacea* were collected in April 2016 in a garden in Sango Ota, Ogun State, Nigeria. The plant was authenticated at the University of Lagos Herbarium (voucher number LUH 6963). The fresh leaves were air-dried indoors for two weeks and crushed using a manual grater. The dried sample was weighed and stored in air tight container.

2.2 Extraction of Plant Materials

The plant materials (100 g) was extracted with n-hexane, ethyl acetate and ethanol by macerating successively in each solvent for 72 h, to obtain the respective extracts. The extracts were concentrated using a rotary evaporator at 40℃. These were further dried using an aerated oven at 40℃ and kept for further use.

2.3 Phytochemical Analysis

Phytochemical analysis was carried out qualitatively and quantitatively. The qualitative phytochemical screening was carried out to determine the presence of secondary metabolites such as tannins, alkaloid, flavonoids, cardiac glycosides, reducing sugar, saponins, and anthraquinones in the different extracts of *Pativeria alliacea* using the standard procedures of Sofowora [12] and Evans [13].

2.3.1 Tannins

About 0.2 g of the plant extract was dissolved in 6 mL of distilled water and then filtered. About 2 mL of the filtrate was pipette into a test tube after which 2 mL of 15% $FeCl_3$ was added. A Blue-black colour indicated the presence of tannins.

2.3.2 Alkaloids

About 0. 2 g of the plant extract was extracted with 20 mL of methanol for 20 min on a water bath and then filtered. To 2 mL of the cold water extract in different test tubes, was added 6 drops of different alkaloids reagents: Dragendorff's. Mayer's and Wagner's reagents. The formation of brownish-red precipitate (Dragendorff's reagent), creamish precipitate (Mayer's reagent) and an orange precipitate (Wagner's reagent) indicated the presence of alkaloid.

2.3.3 Flavonoids

About 5.0 mL of dilute ammonia solution was added to a portion of the aqueous filtrate of each extract followed by the addition of concentrated H_2S0_4. A yellow colouration observed in each extract indicated the presence of flavonoids.

2.3.4 Cardiac glycosides

2.3.4.1 Kedde's test for lactone ring in cardiac glycosides

About 0.5 g of plant extract was boiled with 50 mL of water to obtain a water extract of the plant. The extract was concentrated to dryness and re-dissolved in 10 mL of methanol. To 2 mL of the extract obtained, 1 mL of a solution of 2% of 3, 5-dinitrobenzoic acid in methanol and 1 mL of 5.7% sodium hydroxide were added and the result was recorded after 5 min. The formation of a blue or purple colour indicates the presence of cardenolides.

2.3.4.2 Liebermann-Burchard reaction for steroid/triterpenoid nucleus

About 0.5 g of powder sample was extracted with 50 mL of methanol. The extract was filtered and the filtrate was gently concentrated to dryness on a water bath. 0.2 g of the dried extract was dissolved in 2 mL of acetic anhydride and allowed to cool. With the test tube inside an ice pack and slanted at an angle of about 45 degrees, 2 mL of concentrated tetraoxosulphate (VI) acid was carefully poured by the side of the test tube. Blue-green ring indicates the presence of terpenoids.

2.3.4.3 Keller-Killiani test for de-oxy sugars in cardiac glycosides

A methanol extract was obtained and the extract reduced to dryness. About 0.2 g of this was dissolved in 2 mL of chloroform. Tetraoxosulphate (VI) acid was added to form a layer and a brown ring at the interphase indicates the presence of deoxy sugar (as in cardenolides).

2.3.4.4 Salkowski's test

About 0.2 g of the extract was dissolved in 2 mL of chloroform. Concentrated tetraoxosulphate (VI) acid was carefully added to form a lower layer. A reddish- brown colour at the interface indicate the presence of a steroidal ring.

2.3.5 Reducing sugars

Fehling's test: Water extract of the powdered material was obtained by boiling on the water bath. To 2 mL of the extract, in the test tube was added, 1 mL each of Fehling's solutions A and B. The mixture was shaken and heated in a water bath for 10 min. A brick red precipitate indicates the presence of reducing sugar.

2.3.6 Saponins

Frothing test: Water was added to about 0.2 g of the extract in a test tube. The test tube was shaken vigorously and then left to stand for 10 min. Frothing that persisted on warming indicates the presence of saponins.

2.3.7 Anthraquinone derivatives

Bontragner's test: Chloroform extract of the powdered sample was obtained by boiling on the water bath. To 2 mL of this extract, 1 mL of dilute

(10%) ammonia was added and the mixture was shaken. The formation of brick-pink colour in the upper layer indicates the presence of anthraquinones.

2.4 Quantitative Phytochemical Analysis

2.4.1 Alkaloids

5 g of the dried plant material was weighed into 250 mL beaker and 50 mL of 10% acetic acid in ethanol was added. The mixture was covered and allowed to stand for 4 h. The mixture was filtered into a clean beaker and the extract was evaporated on a water bath to one-quarter of the original volume. Concentrated ammonia solution was added in drop-wise until the precipitation was complete. The solution was allowed to settle and the precipitate was washed with dilute ammonia solution. The mixture was filtered and the residue filtrate (alkaloids) was dried and weighed and the result was calculated as the percentage of the dried plant material [14]. The procedure was carried out thrice to obtain three different results.

2.4.2 Flavonoids

10 g of the dried plant material was weighed into a beaker, 100 mL of 80% aqueous methanol was then added and the plant-solvent mixture was left at room temperature for about 1 h. The whole mixture was filtered through Whatman filter paper No. 42 (125 mm). The filtrate was poured into a crucible and then evaporated to dryness over a water-bath and weighed to a constant weight and the result was calculated as the percentage of the dried plant material [15]. The procedure was carried out thrice to obtain three different results.

2.5 Alpha-amylase Inhibitory Assay

The alpha-amylase inhibitory assay was carried out using the starch-iodide assay and the 3,5-dinitrosalicylic acid (DNSA) assay according to the methods of Sudna et al. [16,17].

2.5.1 Starch- iodine color assay

To 250 µL of the plant extract in DMSO (concentration range 0, 50, 100 µg/mL), was added 250 µL enzyme solution (IU/mL pancreatic alpha-amylase enzyme (E.C:3.2.1.1; Sigma-Aldrich) dissolved in 0.02 M Sodium phosphate buffer with 0.006 M sodium chloride), was incubated for 10 min at 37℃. 250 µL of 1% soluble starch (potato starch) was then added to all the test tubes and incubated again for 10 min at 37℃. This was followed by the addition of 250 µL 1M HCl (hydrochloric acid) to terminate the enzymatic reaction followed by the addition of 100 µL of iodine reagent (5Mm of I_2 and 5Mm of KI). The colour change was noted and absorbance was taken at 620 nm (Thermo Scientific GENESYS 1OS UV/Visible spectrophotometer). The control samples reaction representing 100% enzyme activity did not contain any plant extract. To eliminate the absorbance produced by plant extract, appropriate extract control without the enzyme were also included. Acarbose was used as a positive control at a concentration range of 0, 50, 100 µg/mL The results were expressed as percentage of inhibition of extracts against concentration, calculated according to the formula % Relative enzyme activity = Enzyme of test/Enzyme of control × 100.

% Inhibition in the α-amylase = 100 - % relative enzyme activity [17].

2.5.2 DNSA reagent (3,5-dinitrosalicylic acid) assay

To 250 µL of the plant extract in DMSO (concentration range 0, 50, 100 µg/mL), was added 250 µL enzyme solution (1U/mL of pancreatic alpha-amylase enzyme (E.C:3.2.1.1; Sigma, USA) dissolved in 0.02 M Sodium phosphate buffer with 0.006 M sodium chloride) was incubated for 10 min at 37℃, 250 µL of 1% soluble starch (potato starch) was then added into all the test tubes and incubated again for 10 min at 37℃. The reaction was terminated by adding 500 µL of DNSA reagent (3,5-dinitrosalicylic acid:) and then boiled in the water bath at boiling point for 5 mins. The solution was cooled and diluted with 5 mL of water and absorbance was taken at 540 nm (Thermo Scientific GENESYS 1OS UV/Visible spectrophotometer). To eliminate the absorbance produced by plant extract, appropriate extract control without the enzyme were also included. Acarbose was used as a positive control at a concentration range of 0, 50, 100 µg/mL The results were expressed as percentage of inhibition of extracts against concentration, calculated according to the formula % Relative enzyme activity = Enzyme of test/Enzyme of control × 100.

% Inhibition in the α-amylase = 100 - % relative enzyme activity [17].

2.6 Statistical Analysis

All assays were carried out in triplicates and results were expressed as mean ± standard deviation. All statistical analysis were carried out using excel.

3. RESULTS

3.1 Extraction of Plant Materials

The successive extraction of the plant material with hexane, ethyl acetate and ethanol gave the hexane (1.0%), ethyl acetate (1.0%) and the ethanol (5.2%) extracts respectively.

3.2 Phytochemical Screening

The phytochemical screening results showed alkaloids and cardiac glycosides were detected in all the extracts; saponins, flavonoids, anthraquinones and reducing sugars were detected in the ethyl acetate and ethanol extracts; steroids was detected only in the hexane extract; tannins and terpenoids were not detected in all the extracts (Table 1). All or some of these secondary metabolites may be responsible for the medicinal properties exhibited by *Petiveria alliacea*.

3.3 Quantity of Alkaloids and Flavonoids

The quantity of alkaloids and flavonoids were detected and expressed as percentage of plant materials. The percentage alkaloids and flavonoids in *Petiveria alliacea* leaf are 2,14 ± 0.16 and 6.14 ± 1.07 respectively (Table 2).

3.4 Alpha-amylase Inhibitory Activities

3.4.1 Qualitative assay

The qualitative study of the alpha-amylase inhibitory properties of the leaf extracts of *Petiveria alliacea* was carried out using the starch-iodine colour complex formation assay as described by Sudha et al. [17]. Qualitative alpha-amylase assay gave positive results with the formation of blue complexes in all the extracts of *Petiveria alliacea* (Table 3) initiating a further work to quantify the alpha-amylase inhibitory activities of the extracts.

Table 1. Qualitative phytochemical analysis of *Petiveria alliacea*

S/N	Constituent/ test	PaH	PaEA	PaEt
1.	Tannins	-	-	-
2.	Alkaloids	+	+	+
3.	Flavonoids	-	+	+
4.	Cardiac glycoside			
	Kedde	+	+	-
	Killer-killani's test	+	+	+
5.	Steroidal nucleus			
	Liebermann-burchard	-	-	-
	Salkowski test	+	-	-
6.	Reducing sugar	-	+	+
7.	Saponins	-	+-	+
8.	Anthraquinone	-	+	+

Keyword: PaH= Petiveria alliacea Hexane extract; PaEA = Petiveria alliacea Ethyl-acetate extract; PaEt Petiveria alliacea Ethanol extract, +: Detected; –: Not Detected

Table 2. Percentage composition of the alkaloids and flavonoids and in *Petiveria alliacea*

	% Phytochemical
Alkaloids	2.14±0.16
Flavonoids	6.14±1.07

Result= Mean % ± S.D; n= 3

Table 3. Colour of complex formed from the starch-iodine assay

Extract	Colour change in starch iodide assay
PaH	Dark-blue
PaEA	Dark-blue
PaEt	Dark-blue
Acarbose	Dark-blue
Positive Control (DMSO+starch+Enzyme)	Yellow

3.4.2 Quantitative assay

Alpha-amylase inhibitory properties were quantified in all the extracts using the starch-iodide assay and the DNSA reagent (3,5-dinitrosalicylic acid) assay at two extract concentrations- 50 µg/mL and 100 µg/mL. Both

methods indicated pronounced inhibitory activities by the extracts at 50 µg/mL and 100 µg/mL. In all the extracts, there was an increase in percentage inhibition as the concentration increases from 50 µg/mL to 100 µg/mL (Table 4). The results also showed the extracts to be more potent in inhibiting alpha-amylase than acarbose at both concentrations.

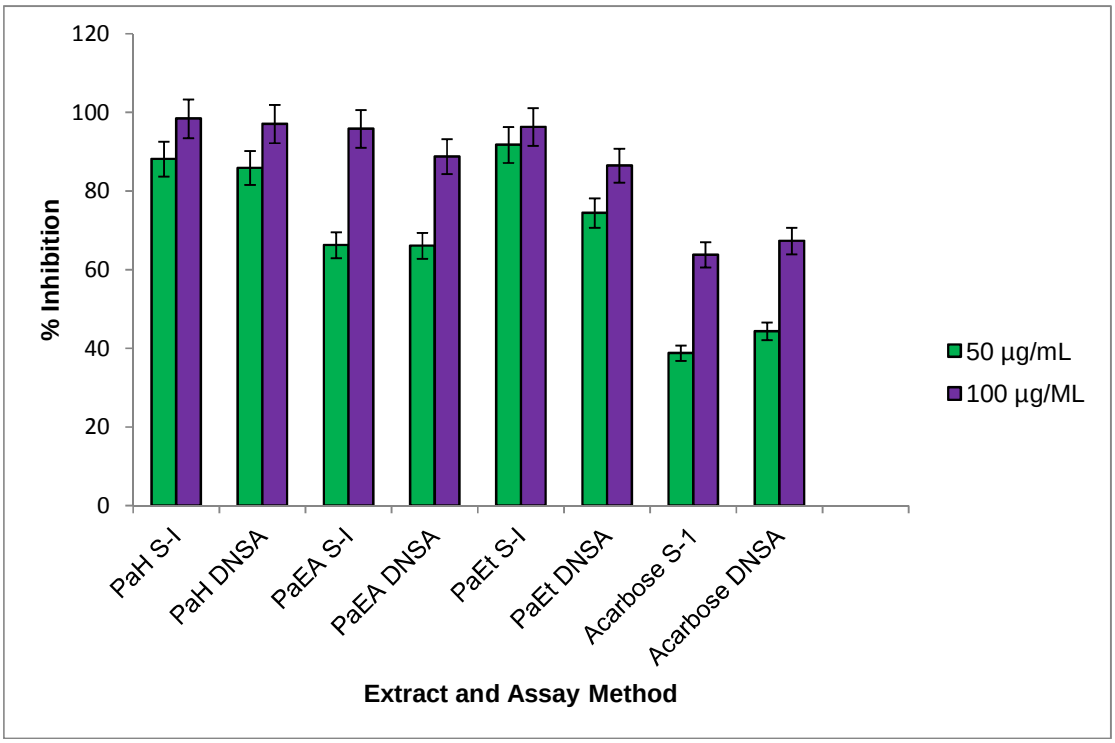

Fig. 1. Comparative percentage inhibition of extracts and the assay method

PaH S-I: Petiveria alliacea hexane extract Starch-Iodide Assay;
PaH DNSA: Petiveria alliacea hexane 3,5- dinitrosalicylic acid Assay;
PaEA S-I: Petiveria alliacea Ethylacetate extract Starch-Iodide Assay;
PaEA DNSA: Petiveria alliacea Ethylacetate extract 3,5- dinitrosalicylic acid Assay
PaEt S-I: Petiveria alliacea ethanol extracts Starch-Iodide Assay;
PaEt DNSA: Petiveria alliacea ethanol extracts 3,5- dinitrosalicylic acid Assay.
Acarbose-S-I: Acarbose starch-Iodine Assay
Acarbose DNSA: Acarbose 3,5- dinitrosalicylic acid Asay

Table 4. Assay methods and the mean percentage inhibition of extracts at 50 µg/mL and 100 µg/mL

Extract	Conc. µg/ML	% Inhibition µg/ML	
		S-1 Assay	DNSA
PaH	50	88.16±1.79	85.90±8.75
	100	98.41±0.75	97.08±0.56
PaEA	50	66.29±2.14	66.11±1.20
	100	95.87±2.17	88.79±5.10
PaEt	50	91.77±4.11	74.44±1.69
	100	96.33±1.21	86.48±0.65
Acarbose	50	38.84±12.20	44.39±14.24
	100	63.83±3.57	67.33±6.59

Key, PaH: Petiveria alliacea Hexane extract; PaEA: Petiveria alliacea Ethylacetate extract; PaEt: Petiveria alliacea Ethanol extracts; S-I Assay: Starch-Iodide Assay; DNSA: 3,5- dinitrosalicylic acid

4. DISCUSSION

Over the years, medicinal plants had served as source of alternative therapy for the management and treatment of several diseases [18,19]. These therapeutic properties of medicinal plants are attributed to their phytochemical contents which vary from one plant to the other [2]. In this study, the preliminary phytochemical screening of the different leaf extracts of *Petiveria alliacea* showed the presence of secondary metabolites which vary in the different extracts. Alkaloids and cardiac glycosides were detected in all the extracts while saponins, flavonoids, anthraquinones and reducing sugars were detected in the ethyl acetate and ethanol extracts; steroids were detected only in the hexane extract; tannins and terpenoids were not detected in all the extracts. Alkaloids had been linked to diverse healing properties exhibited by medicinal plants. Some alkaloids were reported to have anti-diabetic and antioxidant properties [20,21,22]. Cardiac glycosides are known for their cardiotonic properties [23]. Aside from the cardiotonic properties, some cardiac glycosides had also been reported to improve glucose tolerance and ameliorate complications associated with high blood glucose concentrations [24,25,26]. Tannins, flavonoids and saponins are polyphenolic compounds known for their anti-oxidant and anti-diabetic properties [17]. The quantitative phytochemical study indicated a higher percent of flavonoids compared to alkaloids. Flavonoids and other polyphenol compounds are reported to cause reduction in blood glucose due to their ability to induce the regeneration of beta cells which enhances insulin activities [17,27,28,29]. They are also known to inhibit carbohydrate enzymes because of their ability to bind with proteins [30,31]. This may be responsible for some of the therapeutic properties of this plant.

The control of post-prandial glucose concentration in diabetics is a major challenge since high concentration could induce non-enzymatic glycosylation of various proteins, which results in the development of the various complications associated with diabetes mellitus [5,6]. In this study, the various extracts of *Petiveria alliacea* showed alpha-amylase inhibitory properties at both concentrations studied using the starch-iodine and DNS acid assay methods. In the starch-iodine assay, the presence of starch in the assay mixture due to non-degradation when an inhibitor is present is indicated by the formation of a complex between the starch and the iodine forming a dark-blue colour. A yellow colour indicates the absence of starch since there is no starch-iodine complex formed. A partial degradation may occur due to partial inhibitory activity of extracts and this is indicated by the formation of a brown colour [16,17]. In the 3,5-dinitrosalicyclic acid assay, the acid gets reduced to 3-amino-5-nitro salicylic acid in the presence of the free carbonyl group (C=O), present in the reducing sugars, (maltose formed due to alpha-amylase activity on starch), under alkaline conditions [26,31].

Comparing the two assay methods, there is no significant change in the percentage inhibition values obtained in the hexane, ethyl acetate extracts and the standard drug, acarbose (Table 4; Fig. 1). The ethanol extracts showed a higher percentage inhibition values with the starch-iodide assay than the DNS acid assay probably due to other activities.

5. CONCLUSION

The leaf extracts of *Petiveria alliacea* showed alpha-amylase inhibitory properties with higher activities than the control drug, acarbose. The results suggest the plant contain phytochemicals that could effectively reduce post-prandial glucose levels in diabetics. Both methods of assay utilized in this work are effective in the quantitative determination of alpha-amylase inhibition.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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